NATIONAL INSTITUTE OF TECHNOLOGY

DISSERTATION

TITANIA NANOPARTICLES FOR THE INTRACELLULAR DELIVERY OF PACLITAXEL IN BREAST CANCERS

by

RACHNA MUND
(109BM0658)

Submitted in partial fulfilment of the
requirements for the degree of
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Guided by: PROF. AMIT BISWAS

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Certificate

This is to inform that the thesis entitled ***"Titania Nanoparticles for the Intracellular Delivery of Paclitaxel in Breast Cancers"*** submitted by **"*Rachna Mund*" (109BM0658)** in partial fulfilment of the requirements for the award of ***Bachelor of Technology in Biotechnology & Medical Engineering*** at National Institute of Technology, Rourkela is an authentic work carried out by her under my guidance and supervision.

To the best of my knowledge the matter embodied in this thesis has not been submitted to any university/institute for the awarding of any degree/diploma.

Date:

Dr. Amit Biswas,
Department of Biotechnology & Medical Engineering,
National Institute of Technology, Rourkela.

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A HUMBLE SUBMISSION BEFORE YOU ALL

Rachna Mund
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Abstract

Nanoparticle carriers for delivery of anticancer agents enjoys its own unique place due to its stealth nature to body immune system. TiO_2 nanoparticles remain intact in the intercellular and intracellular areas of the cell for clear detection instead of getting digested by the cell. Synthesis of nanocrystalline anatase TiO_2 powders was done by high temperature hydrolysis of $TiOSO_4$ aqueous solution. Morphological characterizations of particle size and shape which are the key features that govern the physical stability of TiO_2 nanoparticles were studied by DLS, NTA, TEM and XRD. Crystalline purity was determined through FT-IR and zeta potential investigation verifies high stability of the nanoparticles.

PCT is conjugated with the TiO_2 nanoparticles because of its favourable mechanism of action in causing dynamic instability during the cell division process of the cancer cells resulting in apoptosis. The efficacy of PCT-TiO_2 nanocomposites is studied on MCF 7 breast cancer cell line.

Drug encapsulation and drug release profile has been performed in vitro at three different pH. The quantitative estimation of amount of drug release, verified through HPLC shows maximum release of 87.197 µg at pH 5.2, 70.023 µg at pH 6.0 and minimum release of 20.341 µg at pH 7.4. This is the most significant outcome in the study for which the pH sensitive PCT-TiO_2 nanocomposite can be claimed as a smart nanocarrier.

To establish the probable mechanism of this TiO_2 nanoparticulate carrier system, a proposed model system is given which is based on the difference in the value of the complexation constant in order of 1 for TiO_2 at pH below 6.7 and above it. Furthermore the stability of this complex and its dissociation is also affected by concentration of Ca^{2+} and capacitance of plasma membrane of normal cell and cancerous cell.

Thereafter a number of biological study has been performed to verify the cellular uptake rate, cytotoxicity, type of death associated with cancerous cells of breast cancer cell line MCF-7. The flow cytometry confirms that the cellular uptake follow a time dependent pattern since there is increase in fluorescence intensity with respect to time up to 24 hours, establishing the fact that accumulation of nanoparticles inside the cells occurs gradually with

time. The MTT assay show synergistic effect of TiO_2-PCT nanocomposite than that of pure PCT over MCF-7 cell line. This study is further supported by the fluorescent pictures of apoptotic cells containing fragmented nucleus and DNA using 4′, 6-diamidino-2-phenylindole staining. Thus there is a possibility of existence of two types of cell death pathway: one is apoptosis dependent and the other normal PCT induced microtubule stabilized cell killing.

It was demonstrated that PCT-TiO_2 nanocomposite increases intracellular concentration of PCT and enhance its anticancer efficiency by inducing the above two mechanism and hence acts as an efficient DDS importing PCT into target cancer cells. These findings suggest that “smart” PCT delivery strategy is a promising approach to cancer therapy.

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List of Abbreviations

Å	angstrom
^{0}C	degree Celsius
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
µg	microgram
µL	microlitre
µm	micrometer
AAV	adeno-associated virus
AIDS	acquired immunodeficiency syndrome
Ag	silver
ATP	adenosine triphosphate
Au	gold
AU	absorbance unit
AUC	area under the curve
Ca	calcium
$CaCl_2$	calcium chloride
$CaPO_4$	calcium phosphate
CO_2	carbon dioxide
COOH	carboxylic acid
cm	centimetre
CDK	cyclin-dependent kinases
CMFP	cyclophosphamide, methotrexate, fluorouracil, prednisone
DAPI	4',6-diamidino-2-phenylindole

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ELSD	evaporating light scattering detector
FACS	fluorescence assisted cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FeO	ferrous oxide
Fe_2O_3	ferric oxide
FT-IR	fourier transform infrared spectroscopy
g	gravitational force
gm	gram
H	hydrogen
HCL	hydrochloric acid
H_2O	water
HPLC	high performance liquid chromatography
H_2SO_4	sulphuric acid
IICB	Indian Institute of Chemical Biology
kV	kilovolt
L	litre
mg	milligram
mL	millilitre
mm	millimeter
mM	millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mV	millivolt
NaOH	sodium hydroxide
NH_2	amine
NH_3	ammonia
NH_4OH	ammonium hydroxide
$(NH_4)_2SO_4$	ammonium sulphate

nm	nanometer
nM	nanomolar
NTA	nanoparticle tracking analysis
OH	hydroxide group
PBS	phosphate buffered saline
PCT	paclitaxel
PDA	photo diode array
pg	picogram
RES	reticuloendothelial system
RNA	ribonucleic acid
rpm	revolutions per minute
SBF	simulated body fluid
$TiCl_4$	titanium chloride
TiO_2	titanium oxide
TiOH	titanium hydoxide
$TiOSO_4$	titanium (IV) oxysulfate
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	ultaviolet
XRD	x-ray diffraction
ZnO	zinc oxide

INTRODUCTION

The high surface energy of nanoparticles permits them to accommodate higher quantity of drug molecules and their small size is advantageous for attaching to multivalent targeting ligands. Once the nanoparticles are attached to the cell it is prelude to uptake because of their relative lack of shear stress. Their ability to bypass the drug resistance mechanism makes it a valuable aid in drug delivery system in an effort to increase the bioavailability of drugs at the intended site of action[1].

Study was conducted on TiO_2 nanoparticles as they are pH sensitive, show a significant tolerance level and have high affinity towards phosphate moieties. TiO_2 exists in three types of phases i.e. rutile, anatase and brookite. The metastable anatase and brookite transform to stable rutile upon heating. Brookite is orthorhombic while the other two phases are tetragonal in shape[2]. Nanosized anatase TiO_2 has large effective surface area that enhances the surface reactions[3]. Titania nanoparticles can be synthesised by various different methods like metal organic chemical vapour deposition, oxidation of $TiCl_4$, sulfate process, chloride process, impregnation, coprecipitation, industrial preparation by plasma spray synthesis, hydrothermal process and sol-gel route. In this study the high temperature hydrolysis of $TiOSO_4$ aqueous solution is adopted to synthesis nanocrystalline anatase TiO_2 powders. In order to ensure its physicochemical properties for drug loading and release as well as penetrating capability to the cancerous cells, several characterization like DLS, NTA, TEM, XRD, FTIR and zeta potential measurement have been performed.

Drug loading on to the TiO_2 nanoparticles was implemented for the drug delivery systems. So far, daunorubicin[4] and doxorubicin[5] are the two drugs that have been loaded on to TiO_2 nanoparticles to form the nanocomposites. To the best of my knowledge no nanocomposite have been formed with paclitaxel (PCT) and TiO_2. PCT is a FDA approved anticancer drug that has been used to alleviate breast, ovarian, lung cancer, head and neck carcinomas[6] and also it is stated as the first-line treatment for advanced carcinoma of the ovary and non-small cell lung cancer. It is specified for the breast cancer treatment after the failure of a combination chemotherapy with doxorubicin or after 6 months of adjuvant chemotherapy[7]. Drug efficiency and release kinetics synopsis obtained by HPLC is the most significant in the study to claim the pH sensitive PCT-TiO_2 nanocomposite as a smart nanocarrier.

Biological activity of the PCT-TiO_2 nanocomposite was corroborated on MCF-7 breast cancer cell line by performing different biological tests like measuring the uptake rate of the cells by flow cytometry, cytotoxicity via MTT assay, and apoptosis analysis was conducted by DAPI staining and flow cytometry.

Chapter 1 throws a detailed knowledge on the nanoparticle properties, significance, applications and its role in drug delivery system. Benefits, advantages and disadvantages of the anticancer drug is also discussed in this section. Chapter 2 is all about the synthesis and characterization of the nanoparticle. The nanocomposite construction and its essential release estimation has been highlighted in chapter 3. The final chapter i.e. chapter 4 concludes the whole work done by carrying out different experiments to study the biological activity of the nanocomposite over MCF-7 breast cancer cell line.

CHAPTER 1: Literature Review

1.1 Introduction

Cancer is the second most leading cause of death worldwide after the cardiovascular diseases. Despite all the assistance from the upcoming modern technologies and efforts in providing an effective therapy, cancer still remains unconquered because of its unpredictable variations inside the body. Cancer can be broadly sorted into three different stages: local, regional and distant[8]. The initial stage of malignant cancer where it is confined only to its primary organ (origin) is described to be the local stage, which by chemotherapy can be curbed and the advancement of cancer towards the surrounding organs can be restricted or else it would proceed towards its second stage i.e. the regional stage affecting the nearby tissues, organs and the regional lymph nodes. Distant stage is the last stage when many lymph nodes from different parts of the body are affected. This type of cancer is recognised as metastatic cancer and there is very less chances of survival in this stage.

Current scenario in India

After the cervical cancer, breast cancer is a growing problem of concern in India. Male breast cancer is rare compared to breast cancer in women. Rajesh Balkrishnan, an associate professor at the U-M schools of Pharmacy and Public Health, from his recent investigation stated that one in 22 women are likely to suffer from breast cancer[9]. 56% Indian patients have large tumours (2-5 cm) in first detection and majority are in grade-III stage, but in the west it is caught in the earlier stage[10]. Even the five-year survival rate is 60% in India where as it is 79-85% in developed countries. Furthermore, the period of developing breast cancer in women in India is a decade earlier to that in western countries. The threat rate is higher in metropolitan cities than in rural areas, however, the survival rate in later is very low. Breast cancer rate is highly observed in women of higher education and in those communities that have adopted westernized lifestyle like Christians, Parsis and women from metropolitan cities, it is low in the Muslim community. Experts say by 2015 breast cancer cases will double[11] to surpass the cervical cancer and become the leading cause of death in Delhi, Bangalore, Mumbai, Chennai, Bhopal, Ahmedabad and Kolkata with the relative proportion ranging from 21.7-28.7%. Number of cases is about 115,000 per year and is expected to rise to 250,000 new cases per year by 2015. Few reports even indicate that breast cancer has already taken the lead.

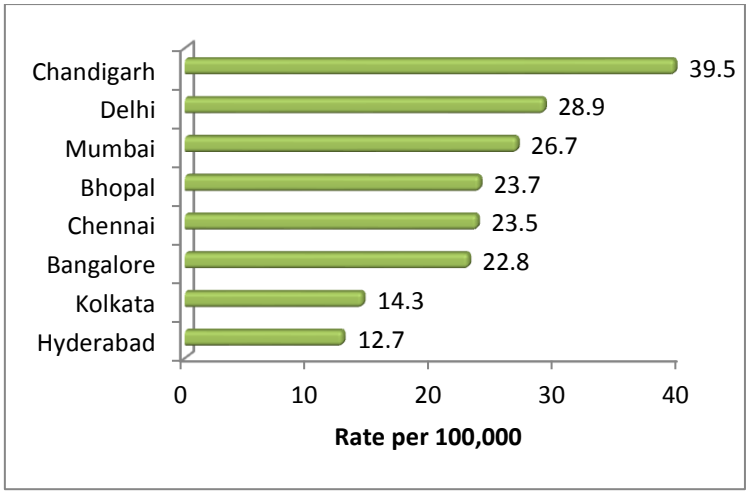

Figure 1: District-wise comparisons of age-adjusted breast cancer incidence rates (per 100,000) under the National Cancer Registry Programme, ICMR, supported by WHO. Source: NCRP, Bangalore.

1.2 Delivery of drugs to tumour sites

The main problem in the anticancer drug delivery is that the drugs affect the healthy tissues the most rather than the cancer cells, thereby increasing the rate of risk. Delivery of drugs should be invoked with selective localization, slow infusions, monitoring of serum drug levels, multi-drug regimens and careful timing[12]. Drugs should reach to the target site with minimal maiming of the healthy tissues. To accomplish these purposes delivery vehicles for drugs come into the limelight. They should be biocompatible, nontoxic, non-immunogenic, biodegradable and be able to recognise and avoid the host's defence mechanism[13]. Chemical conjugation of drugs directly to targeting proteins via the delivery vehicles like liposomes, polymeric micelles, synthetic polymers, dendrimers, nanoparticles, artificial DNA nanostructures, recombinant antibodies or cell-specific cytokines can cause the steric stabilisation, long circulation of particles, controlled release, supplying higher concentration of drugs for extended period encouraging long-term drug delivery.

Unlike liposomes which express low stability, instantaneous uptake and clearance by RES[14], nanoparticles show the greater advantage of being used as the delivery vehicle due to their ability to bypass the clearance by reticuloendothelial system[1]. The high surface energy of nanoparticles permits them to accommodate numerous drug molecules and their small size is advantageous for attaching to multivalent targeting ligands. Once the nanoparticles are attached to the cell it is prelude to uptake because of their relative lack of shear stress. Their

ability to bypass the drug resistance mechanism makes it a valuable aid in drug delivery system in an effort to increase the bioavailability of drugs at the intended site of action[12].

1.3 Delivery of nanoparticles to tumour sites

Targeting of nanoparticles to the site of tumour can be described by two types of mechanisms[15], active targeting and passive targeting. Active targeting is an energy dependent mechanism that involves the conjugation of a tumour specific ligand to the nanoparticle for targeted delivery of drugs. Tumour antigens, tumour vasculature and cell surface receptors act as the target site of tumours, bind with the targeting moieties like antibodies, peptides, cell surface ligands, and aptamers. In preclinical models, targeting has variably led to increased accumulation in tumours. In many instances, cancer cell uptake has been increased with targeting without increase in overall tumour accumulation of nanoparticles.

Passive targeting involves the accumulation of nanoparticles in the endothelium of the tumour blood vessels via their abnormal gap junctions which ranges from 100-600 nm in size, thus making this mechanism an energy independent one. Altered lymph drainage is a characteristic of tumours which favours retention of nanoparticles inside tumours. In general, small particle size is thought to favour intratumoural extravasation. Even though the active targeting mechanism is a much preferred one, the novel approach of delivery of nanoparticle to the site of tumour is still under probation.

1.4 Titania nanoparticles as drug delivery system

Metallic nanoparticles like Fe_2O_3, Ag, ZnO and Au are also used for different therapeutics, diagnosis and treatment of cancer cells, but they come with certain drawbacks like during the production of Fe_2O_3 nanoparticles, FeO nanoparticles are also formed as one is the by-product of the other and it is very difficult to completely isolate the two different nanoparticles and thus their mechanism of action for drug loading and therapeutic activities cannot be assured[16]. Silver nanoparticles on account of their antimicrobial properties have gained much popularity and are extensively used in detergents and wound dressings that end up in the environment in form up waste disposals. It has been reported that at the same concentration ultrafine particles can cause more damage than larger particles[17]. The toxicity of ZnO nanoparticles to T cells fall above 5 mM and the tolerance level to neuroblastoma

cells is above 1.2 mM[18] where as for TiO_2 nanoparticles the tolerance level is above 10 mM[19] and moreover TiO_2 nanoparticles are pH sensitive unlike gold nanoparticles[20].

TiO_2 nanoparticles also have semiconductor properties i.e. when they are excited by UV or near-UV radiation they generate free electrons and free holes with consequent production of reactive oxygen species which are very powerful oxidants and can damage the cell membrane leading to the inactivation of the cell. Thus TiO_2 nanoparticles produce an antimicrobial effect, killing microorganisms or reducing their growth[21].

TiO_2 nanoparticles can also cause inflammation, pulmonary damage, DNA damage and fibriosis[22]. Nanoparticles interact with the immune system by entering into the human stratum corneum. There might also cause an increase in the level of cellular nitric oxide in human bronchial epithelial cells, in addition to the oxidative DNA damage. However, it is reported that titania has a low toxicity[23] and no conclusion of carcinogenicity in humans[24]. Moreover, they remain intact in inter and intracellular areas of the cell for clear detection instead of getting digested by the cell[25]. This makes them an ideal material for safe cancer treatments in humans. Surface defects present in TiO_2 nanoparticles smaller than 20 nm makes them reactive that results in binding with a variety of ligands on the surface. These properties are extremely useful for attaching drugs for an improved drug delivery system, from using TiO_2 as a drug encapsulating agent to using it as a drug surface carrier[4].

1.5 Properties and applications of TiO_2 nanoparticles

Titanium is the ninth most abundant element on earth and titania is a naturally occurring mineral. TiO_2 has been proven to be the most suitable for widespread environmental applications due to its many merits such as TiO_2 possesses good optical and electronic properties, the catalyst is stable, non-toxic, cheap, biologically and chemically inert and insoluble under most conditions, non-radioactive and reusable. It is commercially readily available and cost-effective. In addition to the high surface area nanoparticles and excellent biocompatibility TiO_2 has another unique property of having a high affinity toward phosphate moieties. Also TiO_2 photocatalyst shows a higher photocatalytic activity in comparison with the other photocatalysts.

TiO_2 nanoparticles in aqueous solution form surface hydroxyl groups due to dissociative chemisorption of H_2O molecules on the oxide layer. Depending upon the pH &

ionic environment of the suspension medium the surface groups acquire or release protons; adding to their acid-base properties of the oxide and providing a charge on the surface. This strong redox ability of the TiO_2 nanoparticles makes them useful for photochemical cells, in water or air purification, in degrading the organic pollutants and killing bacteria[26].

They absorb UV light and are used in sunscreen lotions and other cosmetics. They are found as a pigment in powder form and are added to products such as paints, toothpastes, papers, coating plastics, to give them a white colour that lasts years.

Out of the three phases of TiO_2 anatase has higher electron mobility, lower fixed dielectric properties and lower density[27] useful in photocatalytic applications. Other applications in various fields include self-cleaning, gas sensing and sensor devices, dye-sensitised solar cells, electroluminescent hybrid devices, energy-storage technologies, electrodes in lithium batteries and water-splitting catalysts for generating hydrogen.

Types of drugs used for nanocomposite formation with TiO_2

So far to the best of my knowledge, daunorubicin and doxorubicin are the two drugs that have been loaded on to the TiO_2 nanoparticles to form the nanocomposites for the drug delivery systems. The cytotoxic effects, anticancer efficacy enhancement, side effect attenuation of doxorubicin-TiO_2 nanocomposites in human SMMC-7721 hepatocarcinoma cells were investigated by Chen et al and the drug release behavior with respect to different pH values, the tumour cellular uptake along with the cytotoxicity of daunorubicin-TiO_2 nanocomposites in K562 leukemia cells were evaluated by Zhang et al. Both concluded that these two drugs conjugated with TiO_2 nanoparticles hold promising approach as a drug delivery system for clinical practices.

1.6 Paclitaxel

Paclitaxel (PCT) is a highly potent anticancer agent that is obtained by isolating from the bark of the Pacific Yew tree *Taxus brevifolia*. Also known as Taxol, at extremely low concentrations this chemotherapy drug efficiently inhibits cell growth attracting much attention. As reported by Jordan, Toso et al. during the G2-M phase of the cell cycle PCT binds to a region of β-tubulin monomers that hyperstabilizes the microtubules by preventing depolymerization. Dynamic instability creates successful division of chromosomes and cessation of cell division as the metaphase spindle configuration is not achieved. The

prolonged spindle assembly checkpoint causes cell death[20.] Owing to this extraordinary characteristic of the drug, they are treated to kill cancer cells. So far it has been used to alleviate breast, ovarian, lung cancer, head and neck carcinomas[6], but its usage is not limited. For the treatment of advanced carcinoma of the ovary and non-small cell lung cancer, PCT is specified as the first-line treatment. It is specified for the breast cancer treatment after the failure of a combination chemotherapy of PCT and doxorubicin or after 6 months of adjuvant chemotherapy[7].

Even when PCT is a novel antimicrotubule agent, it still comes with certain and major drawbacks like the lack of ability to distinguish between a cancer cell and a normal cell. It destroys the immune system along with causing serious problems in the digestive tract[20]. This is why it should be associated with a carrier vehicle like nanoparticles that would target only the cancer cells and not the healthy tissues. Furthermore, there is no such authenticated clinical marker available that would help predict the sensitivity of the drug[28]. It is insoluble in water due to its hydrophobicity and before administration it must be dissolved in a mixture of cremophor EL and ethanol and unfortunately premedication of steroids and antihistamines are required before PCT administration due to hypersensitivity caused by cremophor EL[29].

Abraxane, the albumin-bound PCT is the available formulation in the market approved by the U.S. Food and Drug Administration for treating breast cancer, ovarian cancer, non-small cell lung cancer and AIDS related Kaposi sarcoma.

Work done with PCT so far

A comparative study of PCT with doxorubicin showed its inferiority; however, it is equivalent to CMFP chemotherapy as front line treatment for patients with metastatic breast cancer[30]. Taxane based neoadjuvant and adjuvant therapy study, prediction of PCT sensitivity by CDK1 and CDK2 activity and correlation of PCT with autophagy in human breast cancer cells, in vivo and in vitro evaluation on the delivery of PCT in improving local therapy for lung and breast cancer by loading the drug onto a polymeric nanoparticle, together with an efficacy comparison with the drug alone, developing peptide-conjugated biodegradable nanoparticles as a carrier to tumour neovasculature and a gold nanoparticle based drug delivery system, conjugating PCT to iron oxide nanoparticles for tumour imaging and therapy, creating formulations of PCT-lipid nanoparticles conjugates and polymeric micelles-PCT conjugates for improved therapeutic and treatment of metastatic breast cancer, incorporation of the drug into EGF-conjugated nanoparticles and even producing a PCT-

conjugated AAV nanoparticles for targeted delivery to breast cells and limiting the exposure of drugs to normal non-breast tissues have already been reported.

1.7 Hypothesis

Drug entrapment can be possible through complexation with transition metal ions. Since transition metals show variable valence with respect to pH of its surrounding environment, it is possible that the drug release can be regulated to site specificity through adjustment of pH in case of the transition metal encapsulating the drug. Therefore, we are in the opinion that TiO_2 nanoparticles loaded with PCT would be able to be directed for cancerous cells as its pH is acidic in nature. Moreover, the release will be further enhanced near lysosomes and vesicles so that the effective toxicity will be enhanced.

CHAPTER 2: Synthesis and Characterization of Titania Nanoparticles

2.1 Introduction

Titania nanoparticles synthesis can be done by various techniques. Industrial preparation contains impurities and degrades the quality of the product (Zhanga T., 2006). Chemical precipitation may give highly pure sol-gel products but it is difficult to control the crystal size distribution[31]. Another procedure is hydrothermal method that includes aqueous or non-aqueous solutions as the starting material and involves the application of elevated temperature and pressure to the solution. TiO_2 nanoparticles obtained from sol-gel route are highly crystalline clear spherical non-homogeneous structures with a diameter of 9 nm, while those obtained via hydrothermal methods are not clear spherical structures, they adhered to one another and agglomeration is more with average particle size is ~19 nm[32]. In this report, the high temperature hydrolysis of $TiOSO_4$ aqueous solutions is adopted to synthesise nanocrystalline anatase TiO_2 powders described below.

2.2 Preparation of TiO_2 nanoparticles

200 mL H_2SO_4 was taken in a beaker and heated inside the fume hood chamber. 80 gm $(NH_4)_2SO_4$ was added periodically to it with continuous stirring. Fumes were produced as the reaction was exothermic. After the complete addition of $(NH_4)_2SO_4$ to the H_2SO_4 it was left for 5-10 minutes and then 10 gm TiO_2 powder was added to the solution in intervals of 10 minutes. After all the TiO_2 powders were added the solution it was left to cool at room temperature for 24 hours. Now the cooled solution was added to 600 mL of distilled water with continuous stirring. After the dilution process was completed the solution was again left to cool at room temperature for 2 hours.

The solution was kept inside the ice bath and 600 mL NH_4OH was added till the formation of a white precipitate takes place. Millipore water was poured for washing and the precipitate was left to settle down. The supernatant was discarded and again the precipitate was washed. This repeated cycle of washing and discarding the supernatant was carried out till the solution's pH reaches 7. Filtration was done and the filtered residues were dried. Later they were transferred to a beaker, sealed and put inside the freezer at -20^0C for 2 hours and then left overnight at -80^0C. After 24 hours of freeze drying, the residues were fired at 700^0C. Dry powders of TiO_2 nanoparticles were obtained and stored at 4^0C.

2.3 Characterization

In order to meet the specific criteria for penetration of nanoparticles and also to be a potential material for drug loading into the cancerous cells, it is required to understand certain physicochemical properties of the nanoparticles that is achieved by the following characterisations:

2.3.1 Dynamic light scattering (DLS)

DLS is a useful tool to determine the agglomeration state of nanoparticles as a function of time or suspending solution. It characterizes the size of a colloidal particle in a solution and measures the hydrodynamic size of a particle. DLS ZS-90 (Malvern, UK) was used here to measure the particle size.

2.3.2 Nanoparticle tracking analysis (NTA)

NTA tracks the Brownian motion, counts and discriminates every particle in the liquid suspension whose speed of motion helps in determining a thorough size distribution analysis. Measurements were performed at room temperature with a NanoSight LM20, equipped with a sample chamber with a 640 nm laser and a Viton fluoroelastomer O-ring. NTA 2.0 Build 127 software was used for capturing and analyzing the data.

2.3.3 Transmission electron microscopy (TEM)

TEM is the most powerful microscope with a significantly higher resolution (by a factor of about 1000) than light-based imaging techniques, providing the quantitative measurement of the particle size, size distribution, and morphological, topographical, compositional and crystalline information. 200 kV TEM JEOL 2000FX was used for the observation of the dried TiO_2 nanoparticles supernatant solution.

2.3.4 X-ray diffraction (XRD)

The phase analysis of the nanoparticles was carried out by XRD. The dried TiO_2 nanoparticle powders were put on the sample holder and characterized by using a Shimadzu, XRD-6000 with Cu K_α radiation (1.5418 Å). Scanning range was set from 20-80^{0}C with scan speed of 2^{0}C/minute.

2.3.5 Zeta potential analyser

Zeta potential determines the interparticle forces in dispersion stability. ZetaSizer Nano ZS (Malvern Instruments) is used to measure the zeta potential of the nanoparticles. In the DTS software the required parameters were entered, temperature set at 25^0C and the data was analysed.

2.3.6 Fourier transform infrared spectroscopy (FTIR)

The chemical composition of any sample can be easily determined by FTIR. Here, FTIR spectrophotometer (Shimadzu) interfaced with IR microscope was operated in reflectance mode. The spectra were acquired in the 4000-400 cm^{-1} region with 8 cm^{-1} resolution, 60 scans and beam spot size of 20-100 μm.

2.4 Results and Discussions

2.4.1 Particle size

Size and shape are the key features that govern the physical stability of TiO_2 nanoparticles, both in vitro and in vivo[33]. The size strongly influences the flow of the particles and their subsequent response to the cancerous tissues. The decrease in particle size results in an increase of the surface area, which may have important implications on the noncrystalline character of the particles and consequently on their flow behaviour. The techniques that are generally employed for the determination of mean particle size and size distributions include DLS, NTA and TEM.

TABLE 2.1: The size distribution measurement of the nanoparticles by DLS

		Size (r.nm)	% Intensity	Width (r.nm)
Z-Average (r.nm): 297.2	Peak 1:	33.14	100.0	2.060
Pdl: 1.000	Peak 2:	0.000	0.0	0.000
Intercept: 1.27	Peak 3:	0.000	0.0	0.000

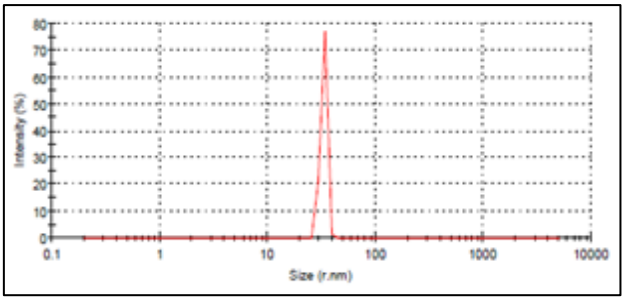

Figure 2.1: Size distribution by intensity obtained from DLS

TiO_2 nanoparticles average size from DLS, was found to be 33.14 nm.

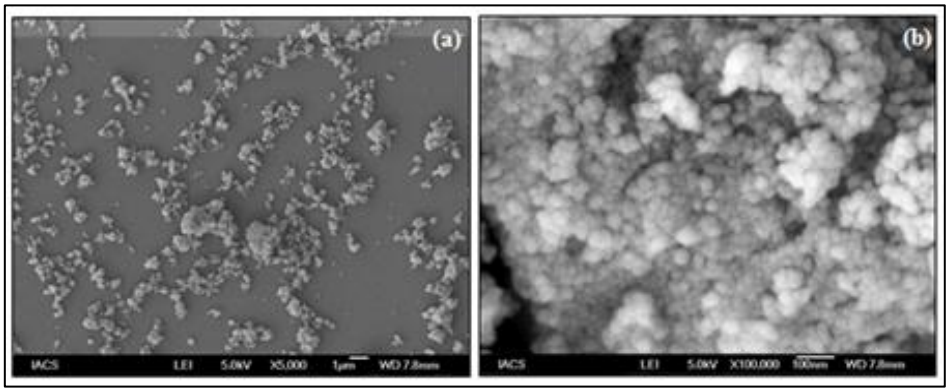

Figure 2.2 TEM images of TiO_2 nanoparticles at scale bar (a) 1µm and (b) 100 nm

TEM images of a small portion of area containing very low particle density showed that the particle size was within 30-40 nm. They are spherical in shape and individual particles do not aggregate among themselves.

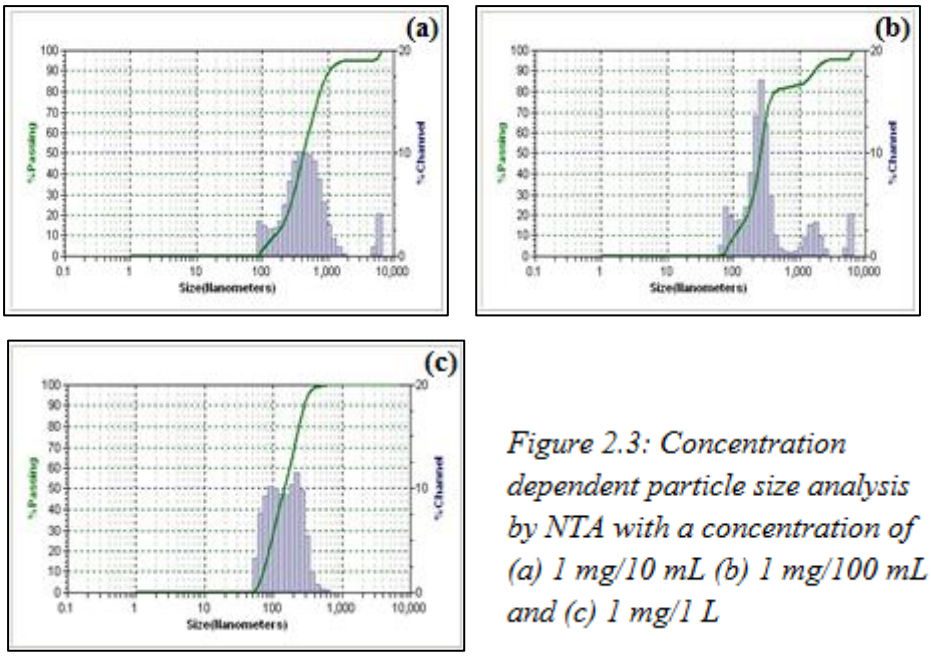

Figure 2.3: Concentration dependent particle size analysis by NTA with a concentration of (a) 1 mg/10 mL (b) 1 mg/100 mL and (c) 1 mg/1 L

NTA results determined more than 50% of particles aggregate in <100 nm dimension. A concrete understanding of the particle size and concentration dependent dimension is able to predict drug loading and encapsulation efficiency and hence helpful in determining the effectiveness of drug-carrier conjugate in site dependent action. Therefore, simultaneous characterization through TEM and NTA is significantly valuable in drug delivery application. Simultaneous measurement of DLS and NTA show that the sample is monodisperse in nature.

Even though DLS and NTA allow the analysis of particle size in a liquid suspension through Stokes-Einstein equation, NTA analyses the suspension on a particle by particle basis[34]. By NTA not only the particle diffusion but also its hydrodynamic diameter can be determined. Whereas, it is not by particle basis in DLS but through a digital correlation time dependent scattering intensity fluctuation. DLS measures the relative Brownian movement of particles through assemblage of large number of particles within a sample. It provides a volume distribution of particle size in contrast to number frequency distribution and relative particle concentration in NTA. NTA is insensitive to contaminants and concentration required is also less than that of DLS.

2.4.2 Particle structure

The structures of composite particles made of TiO_2 and organic molecules such as ligands/drug as well as the nature of the interactions between the TiO_2 core and the associated molecular structures within the composite (e.g., chemical association and/or physical adsorption) have been investigated through FTIR. In this context, the electrophoretic mobility determinations are useful in evaluating the effect of the coatings on the zeta potential (ζ).

The FTIR study provides with the functional groups present in the TiO_2 nanoparticles. 1633 cm^{-1} shows the bending vibration of O-H, 1350–1400 cm^{-1} ranges the CO_2 absorption, the broad band below 900 cm^{-1} is the characteristics of TiO_2 and around 3400 cm^{-1} the stretching vibration of O-H of absorbed water molecule is detected.

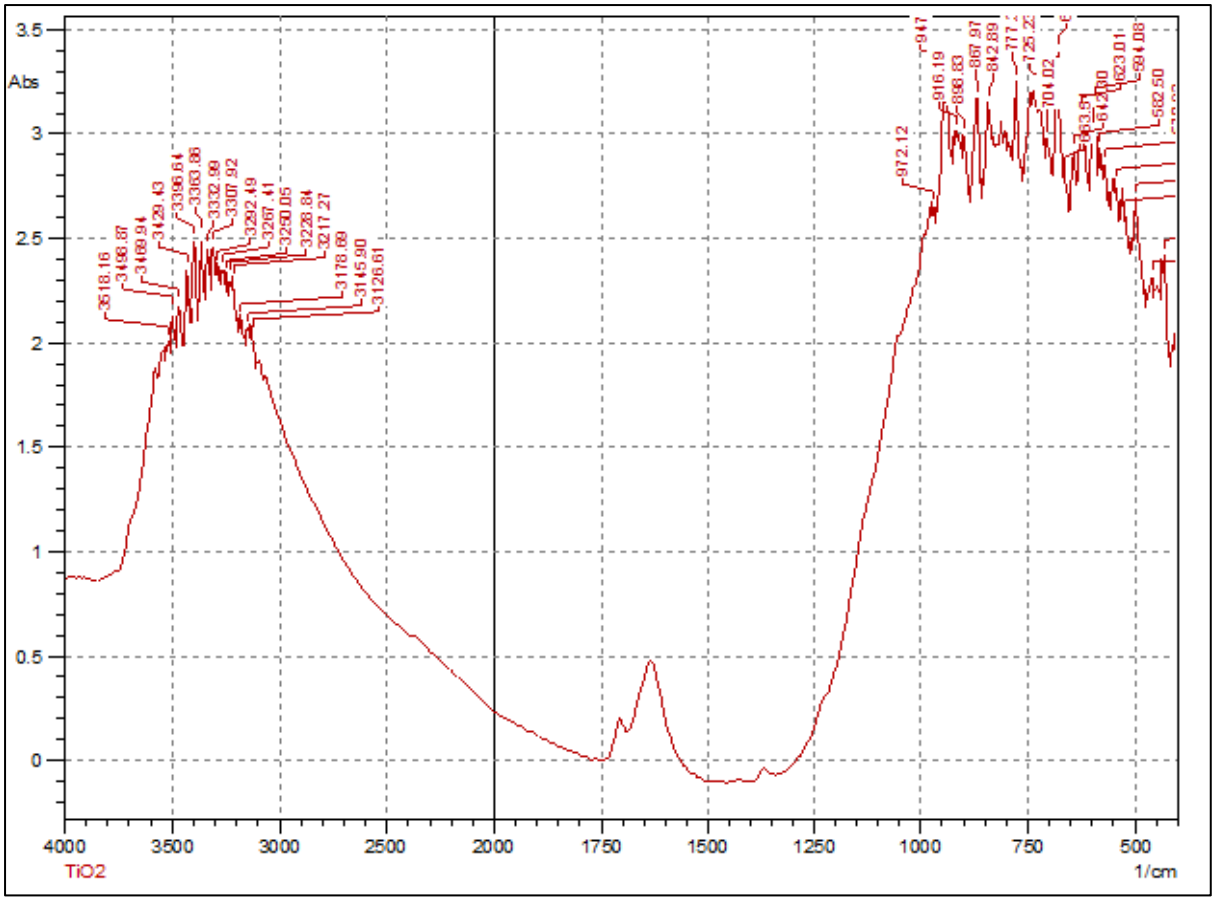

Figure 2.4 FTIR spectra of TiO_2 nanoparticles

The adsorption mechanism of organic additive particles involves mononuclear or binuclear chelation. These bindings are suggested to be occurring via salicylate or catecholate or carbamide type of binding which involves two adjacent OH groups or adjacent COOH and OH or COOH and NH_2 or OH and NH_2 groups providing strong adsorption to form negatively charged particles[35]. Therefore the above study suggests that there will be a high rate of adsorption of PCT over TiO_2 nanoparticles.

2.4.3 Phase analysis

The appropriate mineralogical purity and the degree of crystallinity of TiO_2 define their physiochemical and biological properties. X-ray diffraction can be considered as the most important technique to elucidate the phase of the nanoparticle[33].

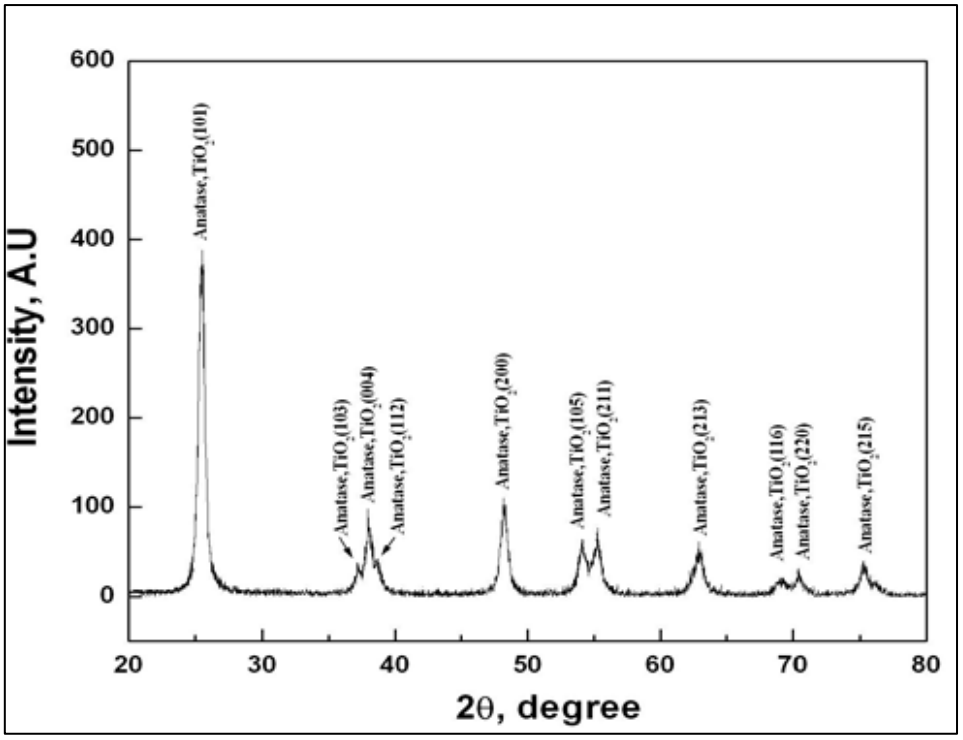

Figure 2.5: XRD profile of TiO_2 nanoparticles

XRD results indicates nearly complete crystallization of TiO_2 nanoparticles. However, retention of some unsharp spots and peaks with surrounding scattered sample indicated slight disorderness of sample. All the identified peaks are proof to be the anatase phase of the TiO_2 and the corresponding diffraction plane are marked in the figure 2.5. The anatase phase of titanium nanoparticle has long been known for its application in implant. It is corrosion resistant that ensures minimum inflammation inside the body. TiO_2 nanoparticles in their anatase phase are important because the protein molecules that bind with the nanoparticles fold themselves inside the body in a way favourable enough to easily escape the clearance by reticuloendothelial system[1] and thus they reside for an extended period inside the body.

2.4.4 Surface charge and stability

The surface properties of nanoparticles are significant in determining the drug carrier potential since they control the interaction with plasma proteins. Zeta potential measurement informs about the overall surface charge of the particles and how it is affected by the environment[33] (e.g. pH, presence of counter ions, adsorption of proteins and its duration of passage with blood flow). The charge shielding by several groups (natural or artificial) may be used for predicting the effectiveness of the barrier function against opsonisation in vivo. Zeta potential can be used to determine the type of interaction between the drug and the

carrier i.e. whether the drug is encapsulated within the body of the particle or simply adsorbed on the surface. This is important because adsorbed drug may not be protected from enzymatic degradation or may be released very rapidly after administration. Its contribution to characterize drug carrier system can be analysed by observing the drug loading and drug release profile. When the zeta potential is greater than +25 mV and lesser than -25 mV the particles have high degrees of stability.

TABLE 2.2: Zeta potential distribution

		Size (r.nm)	% Intensity	Width (r.nm)
Zeta Potential (mV): -28.2	Peak 1:	-28.2	100.0	3.70
Zeta Deviation (mV): 3.70	Peak 2:	0.000	0.0	0.000
Conductivity (mS/cm): 0.00699	Peak 3:	0.000	0.0	0.000

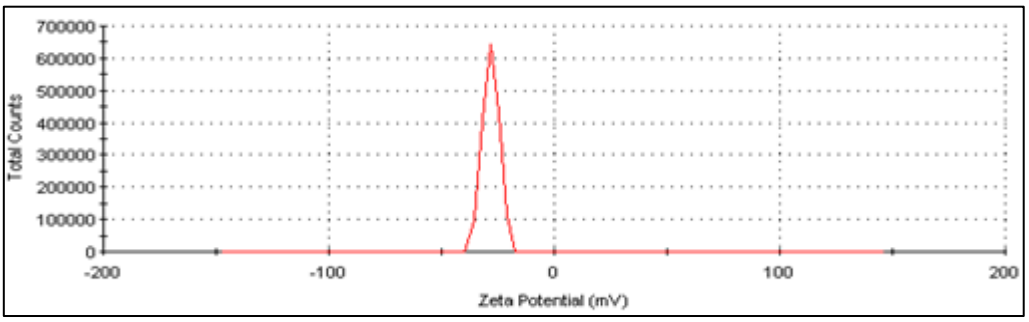

Figure 2.6: Zeta potential distribution

From the Table 2.2 and Fig. 2.6, it was observed that the zeta potential for the nanoparticle was -28.2 mV which indicated greater stability. The influence of pH on the zeta potential is completely different. Polymeric nanoparticles prepared without surfactants have the value close to zero for all but a narrow window around neutrality, indicating their aggregation in acidic or basic medium. On the other hand particles prepared with surfactants had a constant zeta potential irrespective of pH as the surfactant prevented the binding of counter ions within its cavity, so stability can be expected[36].

Salt concentration also affects zeta potential. It also varies from type and molecular weight of the salt and concentration of $CaCl_2$ cause the zeta potential to rise to a plateau at zero which is accompanied by aggregation of nanoparticles. The divalent Ca^{2+} thus acts as a bridging ion but Na^{+} acts as uniquely adsorbed ion which not only screens the zeta potential but reverses it as the concentration of salt increases. So surfactants have little role in this

respect. Hence, the particles are expected to have a positive charge in physiological salt condition and be sensitive to aggregation by divalent cations like Ca^{2+}.

Long circulating nanoparticles are expected to inhibit compliment activation[36]. This zeta potential measurement helps to identify the presence of protein over the nanoparticle and the type of folding that is induced. Drug loading and surfactants can shield the zeta potential of the nanoparticles. Sometimes it may also happen that the negative charge can be shielded to positive that would determine the heavy loading not only by drugs but by other molecules. Thus the behaviour of surface adsorbed drugs can be studied by zeta potential measurement. This zeta potential serve as an important tool for characterizing controlled drug delivery system**,** providing information regarding the surface properties of nanoparticles and therefore determine the organization of the constituent molecules. Incorporation of drug molecules changes the zeta potential indicating the mode of association and its release in different media. It can also be used to follow the adsorption of plasma protein and other molecules onto surface.

CHAPTER 3: Drug Loading, Drug Encapsulation and Drug Release

3.1 Introduction

Drug loading and encapsulation varies with the different drug delivery vehicles like nanocarriers, liposomes, micelles etc. and also with different kinds of drugs. However, the site specificity depends mainly on the type of nanocarrier used. The rate and duration of dissolution of drug depends on the nature of nanocarrier as well as the type of salt crystal and co-crystals used. Use of nanocrystals in conjugation with nanocarriers may enhance the drug dissolution for sparingly soluble and also hydrophobic drugs. This will lead to a reduction in the dosing frequency with improved therapeutic competence since it leads to controlled and sustained release of the drug. An effective and supportive surface functionalization strategy prolongs the therapeutic half-life time in vivo acknowledging circumvention of the immune system and targeted delivery[37]. These advantages indicate use of lower amount of drug, reducing cost and relatively intricate preparation procedure[38]. Nevertheless the greatest advantage of the nanocarrier drug delivery system is decreased patient complicacy due to reduction in dosing frequency with increased tolerability to drugs.

Improvement in analytical system is done to accurately analyse the plasma concentration of blood and correlate these data with the in vitro measurements performed in association with this. In this context, the current study throws light on the construction of the PCT-TiO_2 nanocomposite, drug release mechanisms, drug loading, drug encapsulation and its efficiency measurement by HPLC.

3.2 Drug release by HPLC

Drug release rate at direct real time measurements are extremely essential in understanding and unswerving assessment of its kinetics. This measurement can be done by various techniques out of which high performance liquid chromatography (HPLC) is the most preferable.

It is the most important analytical technique carried out during the drug manufacturing procedures because to ensure a drug's safety and quality intensive and high level of investigation of chemical support at all stages is thoroughly required. In this HPLC

system, the chromatographic dilution of a sample under isocratic elution condition is expressed by the following equation[39]:

$$D = \frac{C_0}{C_{max}} = \frac{\varepsilon\pi r^2(1+k)\sqrt{2\pi LH}}{V_{inj}}$$

Where C_0 is the initial compound concentration in a sample before injection into the HPLC.
C_{max} is the final compound concentration at the peak maximum.
r is the column radius
k is the retention factor
L is the length of the column
H is the column plate height
ε is the column porosity
V_{inj} is the volume of sample injected

3.3 Materials and Methods

3.3.1 Construction of PCT-TiO_2 nanocomposites

2 mL aqueous solution of PCT (0.5 mg/mL) was poured in 1 mL aqueous solution of TiO_2 nanoparticles and three such samples were prepared. The mixture was continuously agitated using a stirrer at 50 g and left overnight in dark to allow the construction of nanocomposites. All the three samples were observed after 12 hours, 24 hours and 48 hours respectively. The nanocomposites formed were separated from the free standing molecules by centrifuging at 5000 g for 30 minutes. The supernatant was determined by high performance liquid chromatography and its loading and encapsulation efficiencies were estimated.

3.3.2 Loading efficiency of drug

Five identical formulations were prepared to find out the loading efficiency of the PCT-TiO_2 nanocomposite. The loading efficiency of a drug is defined as the percentage of ratio of the amount of drug in the nanocomposite formed to the amount of PCT-TiO_2 nanocomposites.

3.3.3 Encapsulation efficiency of drug

Here also five identical formulations were prepared and the encapsulation efficiency was calculated by the percentage of ratio of the amount of drug in PCT-TiO_2 nanocomposites formed to the initial amount of drug used.

3.3.4 Drug release study

The release behaviour of PCT from PCT-TiO_2 nanocomposites was investigated at pH 5.2, 6.0 and 7.4 which are the pHs of endosomes or lysosomes, tumour cells and physiological blood respectively. PCT loaded TiO_2 nanoparticles (20 mg) were dispersed in PBS (pH 7.4, 5 mL) and transferred into a dialysis bag (Pierce, Biotech, thermo Scientific). The dialysis bag was then immersed in 95 mL PBS at pH 5.2, 6.0 and 7.4. The release medium was continuously agitated with a stirrer at 50 g and 37°C. After desired time intervals of 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 36 and 48 hours, 2 mL of the medium was collected and replaced with the same fresh PBS. The amount of released PCT in the medium was then determined by HPLC.

3.3.5 HPLC

Standard drug PCT was purchased from Sigma aldrich. HPLC grade acetonitrile, glacial acetic acid, trifluroacetic acid and hydrochloric acid were procured from Merck Ltd., India. Analytical grade NaOH, hydrogen peroxide and other chemicals used in the study were procured from CDH chemicals Ltd, Mumbai, India. The HPLC system (Shimadzu, Japan) consists of a LC-10AT pump, a SPD-10AVP, PDA detector, Phenomenex C18 (250 mm X 4.6 mm, 5 μm) column, a Phenomenex, HPLC grade cartridge system and a class Nuchrom software. pH of the mobile phase was checked on microprocessor based water proof pH tester (pH tester 20, Eutech instruments, Oakton, USA). The overall illumination at the point of sample placement was tested using a calibrated lux meter (Lutron). Stability study was performed in a hot air oven (oven universal with Thermotech thermostat TIC-4000N, S.M. Industries, New Delhi, India). The mobile phase found to be most suitable for analysis was acetonitrile:phosphate buffer pH 7.4 in the ratio of 60:40. Flow rate employed for analysis was 1.0 mL/min and the concentrations were detected at 226 nm. The concentration of

released sample after predetermined time intervals are quantitatively estimated through HPLC analysis with reference to standard estimation.

3.4 Results and Discussions

3.4.1 Efficiency

TABLE 3.1: Loading and encapsulation efficiency of drug

Formulations	Loading Efficiency (%)	Encapsulation Efficiency (%)
F1	17.34 ± 3.534	62.67 ± 0.884
F2	21.56 ± 5.476	65.12 ± 2.280
F3	18.08 ± 1.299	59.34 ± 18.233
F4	18.94 ± 0.078	67.48 ± 14.977
F5	20.18 ± 0.922	63.45 ± 0.026
Average	19.22	63.61

The efficiency of nanoparticles can be obtained by measuring the loading efficiency of PCT. Study revealed that the average loading efficiency of PCT was 19.22 ± 1.22%. However encapsulation efficiency that determine the affinity of the nanocomposite towards a biological system showed substantially higher efficiency of PCT over TiO_2 nanoparticles i.e. 63.61%. As per the selective diffusion theory, when surface moisture is within 7-23%, the surface of particle acts as a semipermeable membrane and so reduces the diffusion of drugs to the core. This encapsulation efficiency in case of drugs in solution is dependent on concentration, molecular weight and polarity. However, in case of TiO_2 nanoparticles, this is dependent on surface area of the nanoparticles.

3.4.2 Drug release study

The efficiency of drug release can be obtained through release of drug into the outer in vitro medium. The reduction in rate of drug release decreases the diffusivity of a high molecular weight drug like PCT. Study does not explicitly claim a relationship of drug release with respect to the nanocomposite but, however it provides a gross overview regarding the amount of drug released into the cancerous cells.

TABLE 3.2: HPLC drug release study of PCT into SBF at different pHs

Time (hours)	Cumulative release (µg)		
	pH 7.4	pH 6.0	pH 5.2
2	06.511±0.667	19.201±1.661	35.091±1.865
4	10.134±0.912	32.312±1.378	52.824±1.765
6	12.392 ±0.887	41.513±1.993	67.367±2.145
8	14.456±1.239	51.632±2.011	73.452±2.008
10	15.125±1.116	58.159±1.066	76.339±1.854
12	16.306±1.457	60.732±0.966	80.156±1.764
16	17.407±0.995	63.269±0.858	83.336±1.349
20	18.892±1.095	65.335±0.546	84.542±0.987
24	19.134±1.342	65.850±1.662	85.192±0.764
28	19.238±1.056	68.133±0.568	85.344±0.682
36	19.230±0.867	69.254±0.874	86.104±0.943
48	20.341±1.667	70.023±0.483	87.197±1.765

The drug release into the medium estimated through HPLC was plotted in a graph to find the kinetics of release of drug.

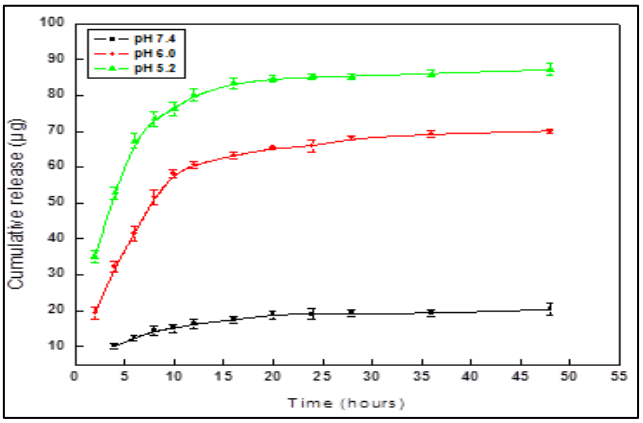

Figure 3.1: Time vs cumulative release of drug at pH 5.2, 6.0 and 7.4

The preparative HPLC is a standard method to determine the amount of drug loading and drug release. The selection of mobile phase should not oxidise/degrade the drug to many other compounds which may interfere in analysis. The compound where degradation kinetics

is very fast, methanol can be replaced for acetonitrile. However, in this case a small peak appeared in the chromatogram after 22 minutes which may correspond to either impurity or a degraded product. Since a large time interval is observed, this peak may correspond to impurities. There may be so many other degraded products formed during the retention time of drug in mobile phase which cannot be detected through the PDA detector. A versatile detector like ELSD can be used for this case but due to lack of facility this turned out to be the limitation in this study. The detection of degraded products holds significant importance due to the fact that early knowledge of the same can be helpful in evaluating stability of the drug and toxicity to patient.

The quantitative analysis of PCT released into the SBF after dialysis through slide-A-lyzer cassette has been analyzed through HPLC. All the 4 steps like sample preparation, assay calibration, sample analysis and data management has been performed sequentially to achieve maximum accuracy with desired precision. The pure PCT (100 ng) has been used as internal standard. The standard curve has been plotted ranging from 1-100 μg and in case of higher concentration, serial dilution technique has been applied to bring the concentration to the desired limit. Every care has been undertaken to minimize the error. The first peak arises after 8 minutes and the AUC (area under the curve) is calculated for a predetermined amount of sample and normalized for all other types of testing sample. The table 3.2 and figure 3.1 represent detailed description of quantitative release of PCT to the in vitro medium.

Study proved the pH specific release of PCT to simulated body fluid at different pH similar to extra cellular fluid. In the case of cumulative release the point of inflexion (mild) occurs after 12-16 hours. After that nearly a stationary phase is maintained up to 48 hours. The difference in drug release rate i.e. normal body pH to cancerous site is nearly 4 times. This indicates the specificity of PCT release effectively near the cancer site. It indicates nearly complete release of within 12 to 16 hours of injection. However the study requires to be verified in the context of in vivo medium in order to find its suitability in presence of various body protein.

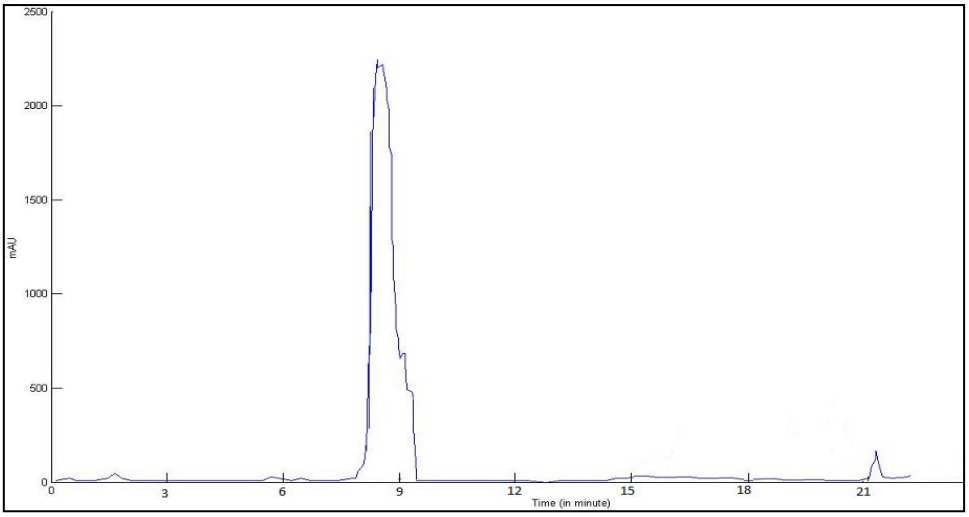

Figure 3.2: Dot plot of HPLC showing PCT release into SBF

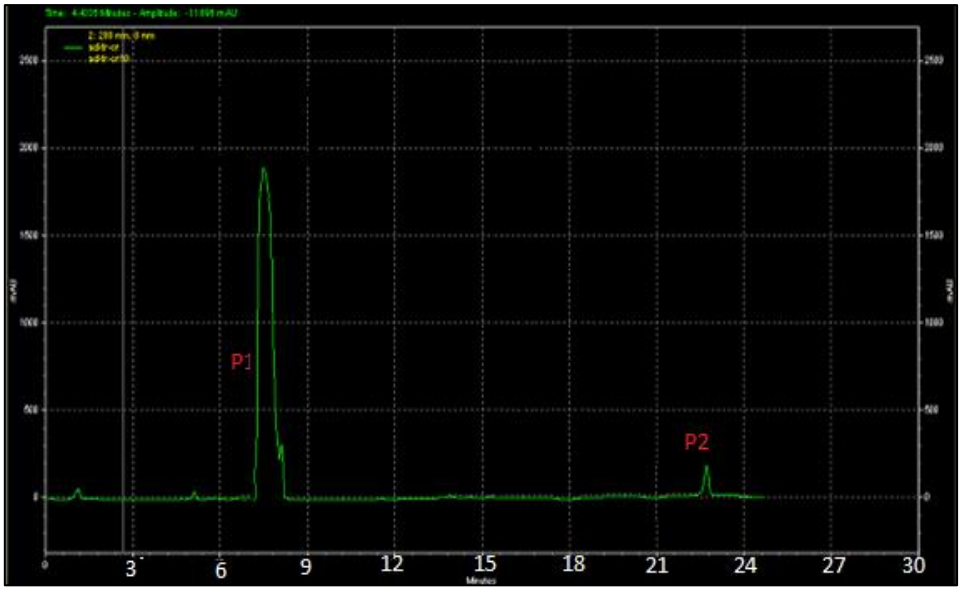

Figure 3.3: Machine generated data of HPLC

Mechanism Of Differential Release:

Titanium is a transition metal with +2 and +4 valencies. The hydrated TiO_2 surface is amphoteric in nature and results in pH-dependent equilibrium between protonated and deprotonated hydrous surface species. TiO_2 nanoparticles obtained from hydrolytic synthesis, if treated with NaOH or NH_3 in water, it will reverse its charge and there will be a certain degree of aggregation. The ionization state of the TiO_2 surface at different pH is as follows:

At pH < 6.7: $TiOH + H^+ \xleftrightarrow{pK_{a1}^s} TiOH_2^+$ (Reaction – 1)

At pH > 6.7: $TiOH + OH^- \xleftrightarrow{pK_{a2}^s} TiO^- + H_2O$ (Reaction – 2)

Below pH 6.7 the TiO_2 nanoparticles remain positively charged. This protonation of drug occurring at low pH releases chemisorbed drug molecules into the medium[40]. Since the nanoparticle is positively charged the electrostatic interaction of PCT and TiO_2 nanoparticles is blocked facilitating drug release. $TiOH^+$ creates random hole in the membrane and hence simultaneous entry of drug and nanoparticle into the cell occurs.

PCT-TiO_2 nanocomposite remains in the blood for a prolonged time period and greatly reduces the side effect to normal tissue. After the creation of hole, the entry of TiO_2 loaded with PCT occurs due to large size of holes. The larger the value of pK_a the smaller is the extent of dissociation at any given pH.

PCT-TiO_2 complexation is favourable and stable at pH > 6.7. Thus the release of drug near normal cells is not possible. Negatively charged TiO_2 acts as a Ca^{2+} scavenger[40,41] along with drug and lipid forming a very stable complex preventing the attachment of TiO_2 into lipid membrane. This complex formation has pK_a value 0-0.58 at pH < 6.7 and 1.858 at pH > 6.7.

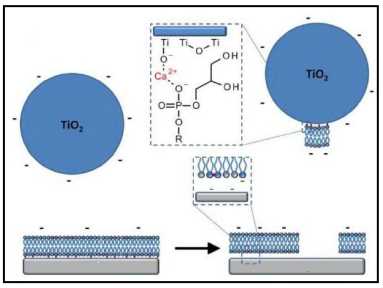

Figure 3.4: In the presence of TiO_2 nanoparticles, Ca^{2+} accumulate at the lipid-TiO_2 interface, leading to the removal of Ca^{2+} ions, as well as whole membrane patches, from the substrate (Zhao et al. 2012).

At the cellular level, $CaPO_4$ is present at the lipid surface. When the TiO_2 nanoparticles reaches the cell surface the Ca^{2+} interacts with the TiO_2 i.e. Ca^{2+} accumulates at the lipid-TiO_2 interface and destabilizes the $CaPO_4$ interaction disturbing the fluid mosaic model. This disturbance reduces the membrane potential and pores are created on the substrate as a whole membrane patch is removed along with the Ca^{2+}. Ion exchange takes place through these pores in order to reach equilibrium and the nanoparticles get released into the cells. Due to the steric hindrance[41] of the $CaPO_4$-TiO_2 complex, the drugs don't get released into the normal cells but this doesn't stop the nanoparticle carrying the drug from penetrating into the cancerous cell.

Then again, there is also the possibility of physical damage to cell membranes by TiO_2 nanoparticles, as the creation of the pores facilitates the entry of TiO_2 into the cell. The photocatalytic properties of nano-TiO_2 under UV irradiation causes photo-oxidative stress that induces the decrease of skin cell stiffness, causes DNA damage, lipid peroxidation and protein damage[42], which if effectively countered by cellular antioxidant defences can prevent cell death. While beneficial for numerous reasons, the design and use of TiO_2 nanoparticles for various applications necessitates a cautious and skilful approach.

CHAPTER 4: Biological activity of PCT-TiO_2 nanocomposite over MCF-7 Breast Cancer Cell Line

4.1 Drug effects on cells

One of the major problems that restricts the efficacy of chemotherapeutic agents used for cancer treatment is the drug resistance capacity of the tumour cells. Even prior to the chemotherapy treatments, tumours may be inherently resistant to the drugs used. It can also happen that initially the tumour cells are sensitive to the drugs and the resistance capacity is acquired during the treatment[43]. This type of resistance is known as acquired resistance. However, such acquired resistance gets exasperating when the tumours also become defiance to other drugs and with different action mechanisms. Over 90% of cases with metastatic cancer is deemed to cause treatment failure because of this innate or acquired resistance faculty of the tumours. If this major problem can be gradually overcome then there would be a significant rise in success rate for the cure of cancer patients.

Mechanisms that influence the tumour environment and regulate drug uptake of tumour cells determine the sensitivity of a drug. The resistivity to chemotherapy can develop at any stage that comprises of various amendments in drug processing like increasing the drug efflux, shifting the target, lowering drug influx, inactivating the drug, administering the damage caused by the drugs and dodging of apoptosis. In this chapter different tests are carried out on the MCF-7 breast cancer cells to have a proper understanding of the tumours' response to the drug and the outcome of these responses.

4.2 Materials and Methods

4.2.1 Cell culture

Maintained MCF-7 breast cancer cell lines were cultured in the cancer culture laboratory with proper safety measures at IICB, Kolkata. In brief, first inside the tissue culture hood, the culture media was prepared that consists of DMEM, 10% FBS, 0.1 mM MEM Non-Essential Amino Acids, 2 mM L-glutamine and 1% Pen-Strep. The freezing medium is composed of 70% DMEM, 20% FBS and 10% DMSO. After the thawing of the frozen cells they were transferred into a vial containing 10 mL DMEM growth media and centrifugation at 1000 rpm for 5 minutes was done at room temperature. The growth media

was removed by aspiration and the cells were resuspended into a fresh growth media (15 mL) by pipetting up and down. The 15 mL cell suspension was then kept in a 37^0C incubator at 5% CO_2. The cell density was monitored daily and was passaged on attainment of 95% confluence .

4.2.2 Cellular internalization

In a six-well culture plate MCF-7 breast cancer cells (1×10^5/mL) were seeded and grown overnight. The wells were divided into four blocks i.e. each for control, TiO_2 nanoparticles, PCT and PCT-TiO_2 nanocomposites and kept in incubator for 6 hours. By using a fluorescent microscope at λem - 515 nm and λex - 488 nm, the cells were examined. Furthermore, the cells were washed 3 times and resuspended in PBS. Analysis of drug uptake by the cells was observed by FACS Aria™ flow cytometer (BD Biosciences, San Jose, CA).

4.2.3 Assay of anticancer activity

The measure of the viability of the cells is done by MTT assay. It is a colorimetric assay that reduces to an insoluble, colored formazan product when it passes the mitochondria inside the cell. This reduction occurs only in metabolically active cells thus determining cell viability. In a 96-well plate the MCF-7 breast cancer cells (1×10^5/mL) were seeded and incubated for 24 hours. Here too the wells were divided into four blocks i.e. each for control, TiO_2 nanoparticles, PCT and PCT-TiO_2 nanocomposites and further incubated for 48 hours. About 20 µl of MTT was added to each well, again incubated for 4 hours and relative anticancer activity was assessed. DMSO was added to solubilize the formazan crystal, and optical density at 595 nm was recorded.

$$\textit{Cell viability (\%)} = \textit{Optical density}_{(595\ nm\ in\ test\ cells)} / \textit{Optical density}_{(595\ nm\ in\ control\ cells)} \times 100$$

4.2.4 DAPI staining

The sample was equilibrated with PBS and the DAPI stock solution was diluted to 300 nM in PBS and 300 µl of this dilute DAPI staining solution was added to the coverslip preparation so that all the cells are completely covered. After incubation for 5 minutes the sample was rinsed thrice with PBS. Excess buffer was removed from the coverslip, mounted and viewed under the fluorescence microscope.

4.2.5 Apoptosis through FACS

To estimate the proportion of MCF-7 breast cancer cells in different phases of cell cycle affected by PCT, cellular DNA contents and apoptosis were measured by flow cytometry as described by Ormerod. 70% of ethanol in PBS was taken and this solution was put drop by drop on an approximately 1 x 10^6cells/well and fixed gently. The whole system was kept in ice overnight. 40 pg/mL propidium iodide and 0.1 mg/mL RNAse were put in PBS and the cell sample solution was resuspended in it. At 37^0C the cells were incubated for 30 minutes and later analysis was carried out on flow cytometry (Becton-Dickinson, San Jose, CA, USA) that is equipped with an argon ion laser at 488 nm wavelength. Cell cycle study along with apoptosis analysis has been performed for determining the proportion of apoptotic cells.

4.3 Results and Discussions

4.3.1 Cell internalization study

Flow cytometry has been employed to quantify the PCT uptake by MCF-7 breast cancer cells. As shown in figure 4.1, the time dependent uptake of PCT-TiO_2 nanocomposites gradually increases with time.

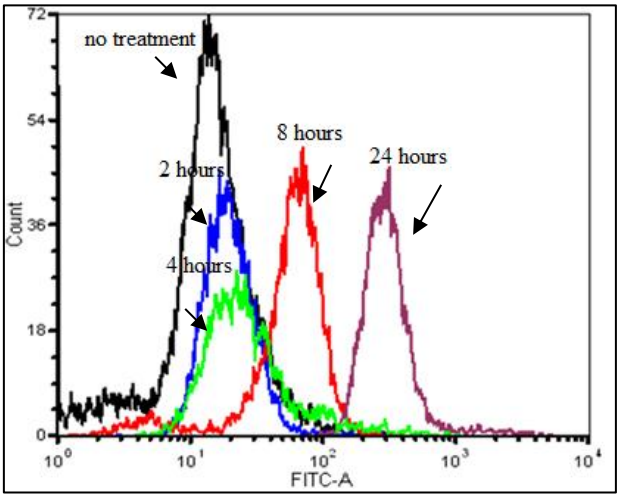

Figure 4.1: Quantification of PCT-TiO_2 nanocomposite uptake by MCF-7 breast cancer cells by flow cytometry

After 2 hours the uptake of nanocomposite shows a logarithmic increase. Thereafter, a steady state increase has been found which does confirms an active uptake of PCT-TiO_2 nanocomposite in comparison to PCT alone which shows passive absorption by the cancer cells. The relative intracellular fluorescence intensity of PCT-TiO_2 nanocomposite quantitatively increases at an order of 2 after 24 hours. Hence the efficacy of TiO_2 nanoparticles as a drug carrier has been confirmed and thereby it represents a promising approach in cancer therapy.

Similar results have been obtained in the study by Chen et al.[4] where nearly 65% increase in fluorescence intensity has been obtained. However this study significantly differs from the results obtained by them. The reason may be a Ca^{2+} mediated mechanism where interaction between Ca^{2+} and TiO_2 may physically and biochemically affect the internalization. The factors which affect this process are likely to be Ca-TiO_2 complexation constant as well as low pH outside cancerous cells which favours the uptake of nanocomposite.

4.3.2 Cytotoxicity study by MTT assay

MTT assay is a very good indicator for understanding the extent of cancerous cell response to PCT-TiO_2 nanocomposite. The assay was performed after 48 hours of cell culture. In case of control and treatment with TiO_2 nanoparticles, cancerous cells show statistically no difference up to a concentration of 1 µg/mL. However, the cell viability of MCF-7 breast cancer cells treated by PCT and the nanocomposite shows drastic decrement. It is to be noted that PCT-TiO_2 nanocomposite reduces the cell viability more than that of cells treated with PCT alone.

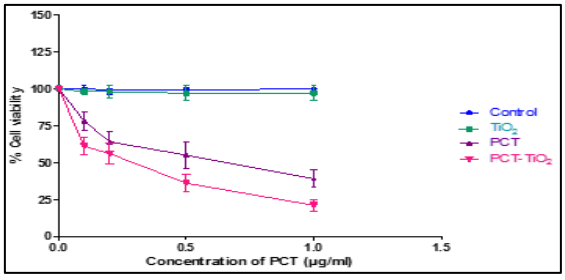

Figure 4.2: Cytotoxicity effect of PCT and PCT-TiO_2 nanocomposite on MCF-7 breast cancer cells by MTT assay

Since TiO_2 treated cells show a survivability of nearly or more than 95%, the non-cytotoxicity of TiO_2 nanoparticles at a concentration up to 1 µg/mL is assured. This lack of

cytotoxicity is the key point for which we have employed this as a carrier for cancer therapy. Increasing concentration of PCT leads to increased lethality to cancerous cells suggesting a dose dependent effect in vitro. Furthermore, the increased cytotoxicity of the PCT-TiO_2 nanocomposite may be due to improved PCT cellular uptake which is supported by an induced release of the drug from nanocomposite within that microenvironment.

The process of controlled release is an interesting phenomena for achieving PCT delivery loaded on TiO_2 nanoparticle at normal physiological pH 7.4. It is assumed that most PCT remains in a conjugated form with TiO_2 and thus serves as a carrier for an extended time period.

Nanoparticle based drug delivery systems may be internalized through endocytic pathway[44]. The endocytosis is a pH dependent phenomena and the occurrence of low pH protonates the drug triggering the release of sorbed drug molecules and turning the surface charge of TiO_2 nanoparticles to positive. This mechanism is further supported by the blocking of electrostatic interaction between PCT and TiO_2 nanoparticles through Ca^{2+} which facilitates the drug release process. This above phenomena has been explained in detail previously. The faster release of the drug from PCT-TiO_2 nanocomposites occurs after the entry of the carrier into the cancerous cell thereby significantly improving the cell killing rate and decreasing the side-effects of the drug i.e. destroying the immune system along with causing serious problems in the digestive tract.

4.3.3 Apoptosis

Cell deaths can be broadly divided into apoptosis and necrosis, the processes differing in their active participation of cells. The term apoptosis originates from the Greek word “falling off” of leaves from a tree, it is used to describe a phenomenon in which a cell actively participates in its own destructive processes. The signal transduction pathway leads to apoptosis and can be defined as an organized physiological mechanism for destroying injured and abnormal cells. This is an important part of cellular processes of cell cycle. There are several anticancer drugs like PCT which at optimal doses in vivo arrest the cancerous cells in their cell cycle.

DAPI staining was performed to find out the improvement in anticancer activity of the pH responsive PCT-TiO_2 nanocomposite. The apoptotic cells are morphologically characterized and evaluated.

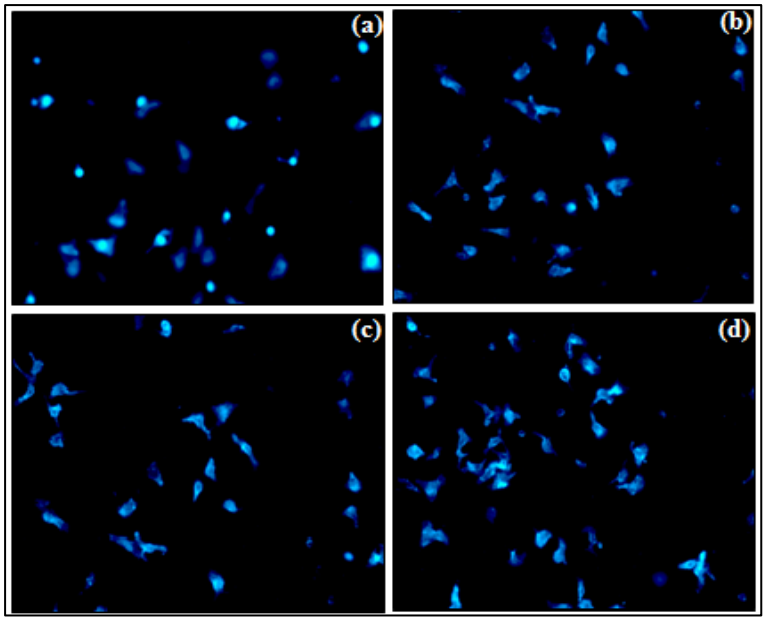

Figure 4.3: Nuclear morphological changes in MCF-7 cells after different treatments for 48 hours (a) untreated cells as control, (b) TiO_2 nanoparticles, (c) PCT alone and (d) PCT-TiO_2 nanocomposite

Morphological analysis shows convoluted nuclei with cavitation and fragmentation, with the formation of apoptotic bodies in MCF-7 breast cancer cell lines. We failed to observe the formation of apoptotic bodies with no treatment and also with TiO_2 nanoparticles conduct. The amount of apoptotic bodies formed upon action with the PCT-TiO_2 nanocomposite is more than that of PCT treated alone. Moreover, we have observed the apoptotic cells treated with the nanocomposite without DAPI staining too.

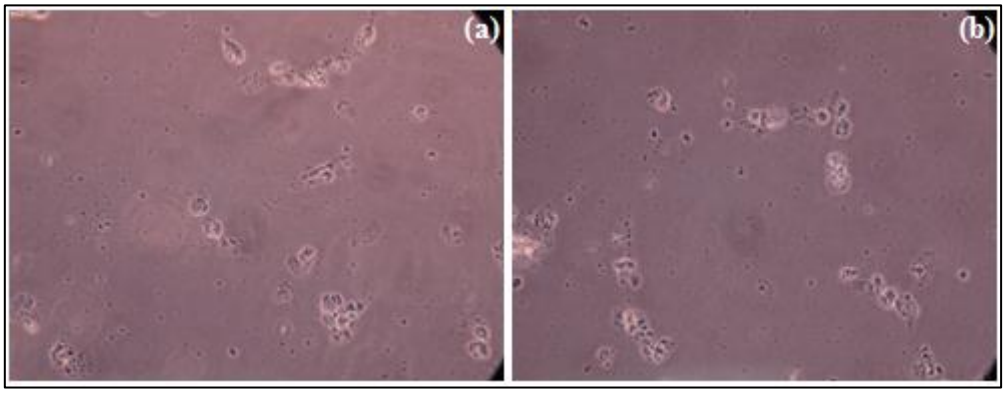

*Figure 4.4: Morphological characterization of apoptosis(**Without Dapi**) in both the images (a) and (b)*

The apoptotic process is characterized with several morphological features i.e. loss of plasma membrane symmetry and its attachment, condensation of cytoplasm and nucleus, and internucleosomal cleavage of DNA. All these phenomena leads to formation of apoptotic

bodies which are rapidly eliminated by phagocytic cells without any inflammatory damage to surrounding cells.

We used flow cytometry assay for cell cycle and apoptosis and it was concluded that PCT could interfere in cell cycle at G_2/M phase, the data corresponding to the G_2/M cycle arrest and thereby inducing apoptosis in MCF-7 cancer cell in dose and time dependent manner. However literature data supports that PCT could induce apoptosis through activation of caspase-3[45].

Pathways initiating apoptosis: Apoptosis results from two alternative pathways: one that is mediated by death receptors on cell surface (extrinsic pathway) and another caused due to changes in mitochondrial function (intrinsic pathway). Both the pathways are regulated by caspases. The extrinsic pathway involves the recruitment and activation of procaspase-8 by receptors containing the death domain located at the cell surfaces. Binding of procaspase-8 to death domain containing receptors results in apparent autocatalytic liberation of active caspase-8, which then activates procaspase-3. Consequently it has been proposed that the active caspase-8 cleaves Bid, generating a fragment that triggers intrinsic pathway. The intrinsic pathway is initiated by release of cytochrome C and other polypeptide from mitochondrial intermembrane space. This release involves the opening of a pore in mitochondrial membrane or translocation of Bcl-2 family membrane from the cytoplasm to mitochondrial membrane. Bax and Bak, in particular have been observed to translocate to mitochondria where they oligomerize to form pores and mediate cytochrome C release that accumulates in the cytoplasm where it binds to the scaffolding protein, Apaf-1, causing an ATP or dATP-dependent conformational change that allows Apaf-1 to bind procaspase-9. This interactivates caspase-9 which subsequently activates caspase-3. The effector caspases 3, 6 and 7 when activated, cleave the key structural components of cytoskeleton and nucleus which results in characteristic morphology of apoptotic cells.

However, this is the limitation of this study as the caspase-3 activity could not be investigated.

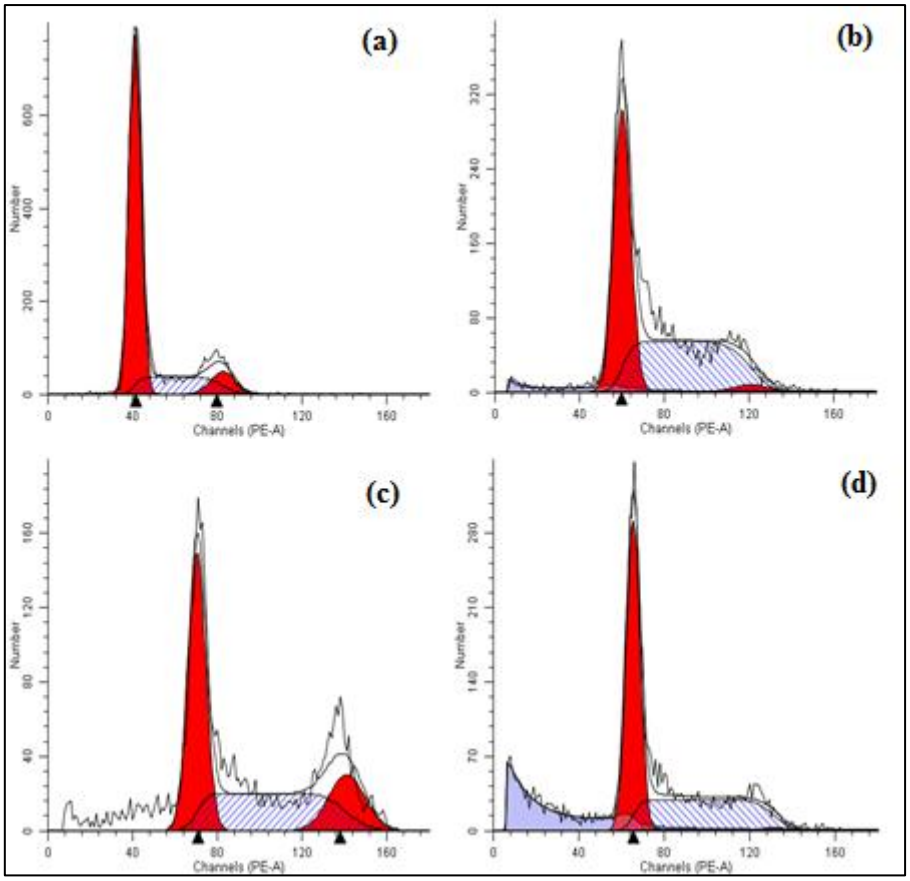

Figure 4.5: MCF-7 breast cancer cells (a) untreated (b) treated with TiO_2 nanoparticles (c) with PCT alone and (d) with PCT-TiO_2 nanocomposite, for 24 hours, washed and then harvested. The cells were fixed and stained with propidium iodide and the DNA content was analyzed by FACS

The figure 4.5 corresponds to the identification and quantification of apoptotic cells through DNA fragmentation. In control i.e. figure 4.5 (a), the cells are in active state of division spreading over G_1, M and G_2 phase with no apoptotic signal. The cells treated with TiO_2 nanoparticles show minimum amount of apoptotic cells as in figure 4.5 (b). This may be due to mechanical rupturing of the membrane of cancerous cells. PCT treated cells, as in figure 4.5 (c), show a typical pattern of DNA content that reflect G_0/G_1-, S- and G_2/M phase of cell cycle together with a sub G_0/G_1 phase corresponding to apoptotic cells by flow cytometry. This sub G_0/G_1 phase show significant increase for MCF-7 breast cancer cell lines upon treatment with the PCT-TiO_2 nanocomposite as is clear from the figure 4.5 (d). This indicates the importance of the nanocomposite over PCT alone.

Overall the MTT assay and FACS analysis of cell cycle shows increased apoptosis of cancerous cells in a concentration and time dependent manner.

The principle followed for apoptosis detection is fragmentation of DNA in correspondence with the cell cycle analysis via flow cytometry. This DNA fragmentation occurs at a later stage of apoptosis due to the activation of endonucleases during the apoptotic program. These nucleases degrade the higher order chromatin structure into 800 Kb fragments and subsequently into smaller DNA pieces. Flow cytometry allows simultaneous consideration of several parameters including size, granularity, and different fluorescent labelling of the same cells detect their pH change[46]. However, this method is non-specific, quick, simple to perform and applicable to all cell types.

Endonucleases are more or less caspase activated DNAse[45] resulting in DNA cleavage and generating broken DNA strands. Since the presence of broken DNA strands in not a unique feature of apoptosis, this assay should be followed by other methods to show specificity of measurement e.g. TUNEL assay.

CONCLUSION

Nanoparticles are useful in improving the solubility of hydrophobic drugs, improving pharmacokinetics through sustained release after biodistribution, protecting sensitive drugs from low pH environment or enzymatic alterations and also most importantly providing targeted drug delivery for desired tissues. The use of functional nanocarriers like TiO_2 nanoparticles provide controlled intracellular delivery of drugs. Here, a functional pH responsive TiO_2 nanoparticles are developed for intracellular delivery of PCT. The pH responsiveness of TiO_2 nanoparticles occur because of two oxidation states i.e. TiO^- and $TiOH^+$ existing above and below pH 6.7 respectively.

Thereafter, the nanoparticles were evaluated for delivery of PCT in vitro to improve local therapy of breast cancer. TiO_2 nanoparticles were synthesized via hydrothermal method and characterized. PCT was successfully encapsulated within the nanoparticles and it was found that the drug release at pH 5.2 is much higher than that at pH 7.4. The uptake of nanoparticles were observed using flow cytometry over MCF-7 breast cancer cell lines in vitro. The potency of PCT loaded nanoparticles was higher than that of the free drug demonstrating a synergistic effect.

The importance of the work lies in the increased bioavailability of drugs at the intended site of action with the development of PCT-TiO_2 nanocomposite for intracellular delivery. Upon injection the nanoparticles remain stable as they encounter physiological pH 7.4. Following the entry into the cells, protein pumps on the membrane of endosome causing the pH inside to drop to a value of pH 5.2. The drug release at this pH into the cytoplasm provides greater efficacy and reduced side effects.

Although the FDA approved drug, PCT is useful in treatment of cancer, this nanocomposite could be beneficial for other cancer types with variation in formulation. Broadening the reach of this PCT-TiO_2 nanocomposite may be a key step in oncologic success. Continued investigation of functional TiO_2 nanoparticles like the system described, will facilitate in the development of new material to meet the requirements for chemotherapy.

REFERENCES

1. Neuberger, T.; Schöpf, B.; Hofmann, H.; Hofmann, M.; von Rechenberg, B., Superparamagnetic nanoparticles for biomedical applications: possibilities and limitations of a new drug delivery system. *Journal of Magnetism and Magnetic Materials* **2005,** *293* (1), 483-496.
2. Kolen'ko, Y. V.; Burukhin, A. A.; Churagulov, B. R.; Oleynikov, N. N., Synthesis of nanocrystalline TiO_2 powders from aqueous $TiOSO_4$ solutions under hydrothermal conditions. *Materials Letters* **2003,** *57* (5), 1124-1129.
3. Peng, T.; Zhao, D.; Dai, K.; Shi, W.; Hirao, K., Synthesis of titanium dioxide nanoparticles with mesoporous anatase wall and high photocatalytic activity. *The Journal of Physical Chemistry B* **2005,** *109* (11), 4947-4952.
4. Zhang, H.; Wang, C.; Chen, B.; Wang, X., Daunorubicin-TiO_2 nanocomposites as a "smart" pH-responsive drug delivery system. *International journal of nanomedicine***2012,** *7*, 235.
5. Chen, Y.; Wan, Y.; Wang, Y.; Zhang, H.; Jiao, Z., Anticancer efficacy enhancement and attenuation of side effects of doxorubicin with titanium dioxide nanoparticles. *International journal of nanomedicine* **2011,** *6*, 2321.
6. 6. 34. P.B. Schiff, J. Fant, S.B. Horwitz, *Nature* 277 (**1979**) 665–667.
7. Rybak, M.; Lomaestro, B.; Rotschafer, J. C.; Moellering, R.; Craig, W.; Billeter, M.; Dalovisio, J. R.; Levine, D. P., Therapeutic monitoring of vancomycin in adult patients: a consensus review of the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists. *American Journal of Health-System Pharmacy* **2009,** *66* (1), 82-98.
8. Zubris, K. A. V., *Polymeric nanoparticles for the intracellular delivery of PCT in lung and breast cancer*. 2011.
9. http://timesofindia.indiatimes.com/life-style/health-fitness/health/Breast-cancer-survival-rates-low-in-rural-India/articleshow/18351175.cms?intenttarget=no
10. http://ogaancancerfoundation.org/sapphire/main.php?url=/
11. http://www.dnaindia.com/health/1600847/report-breast-cancer-cases-in-india-to-double-by-2015-experts
12. Torchilin, V., Micellar nanocarriers: pharmaceutical perspectives. *Pharmaceutical research* **2007,** *24* (1), 1-16.

13. Scott, R. C.; Crabbe, D.; Krynska, B.; Ansari, R.; Kiani, M. F., Aiming for the heart: targeted delivery of drugs to diseased cardiac tissue. **2008**.
14. Seidman, A.; Hudis, C.; Pierri, M. K.; Shak, S.; Paton, V.; Ashby, M.; Murphy, M.; Stewart, S. J.; Keefe, D., Cardiac dysfunction in the trastuzumab clinical trials experience. *Journal of clinical oncology* **2002,** *20* (5), 1215-1221.
15. Grobmyer, S. R.; Iwakuma, N.; Sharma, P.; Moudgil, B. M., *What is cancer nanotechnology?* Springer: 2010.
16. Wahajuddin, S. A., Superparamagnetic iron oxide nanoparticles: magnetic nanoplatforms as drug carriers. *International journal of nanomedicine* **2012,** *7*, 3445.
17. Asharani, P.; Wu, Y. L.; Gong, Z.; Valiyaveettil, S., Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology* **2008,** *19* (25), 255102.
18. Rasmussen, J. W.; Martinez, E.; Louka, P.; Wingett, D. G., Zinc oxide nanoparticles for selective destruction of tumour cells and potential for drug delivery applications. *Expert opinion on drug delivery* **2010,** *7* (9), 1063-1077.
19. Ghosh, M.; Bandyopadhyay, M.; Mukherjee, A., Genotoxicity of TiO_2 nanoparticles at two trophic levels: Plant and human lymphocytes. *Chemosphere* **2010,** *81* (10), 1253-1262.
20. Gibson, J. The development of gold nanoparticle conjugates for the intracellular delivery of PCT. RICE UNIVERSITY, 2010.
21. D.M. Blake, P. Maness, Z. Huang, E.J. Wolfrum, J. Huang, W.A. Jacoby, Sep. Purif. Methods 28 (1999) 1.
22. R. Dunford, A. Salinaro, L. Cai, N. Serpone, S. Horikoshi, H. Hidaka, J. Knowland, FEBS letters 418 (1997) 87-90.
23. Boffetta, P.; Soutar, A.; Cherrie, J. W.; Granath, F.; Andersen, A.; Anttila, A.; Blettner, M.; Gaborieau, V.; Klug, S. J.; Langard, S., Mortality among workers employed in the titanium dioxide production industry in Europe. *Cancer Causes & Control* **2004,** *15* (7), 697-706.
24. Baan, R.; Straif, K.; Grosse, Y.; Secretan, B.; El Ghissassi, F.; Cogliano, V., Carcinogenicity of carbon black, titanium dioxide, and talc. *The Lancet Oncology* **2006,** *7* (4), 295-296.
25. Hu, S.; Yan, Y.-H.; Wang, Y.-F.; Cao, X.-Y.; Li, S.-P., Titanium Dioxide Nanoparticle Absorbed by Hepatoma Cells in Vitro. *Journal of Wuhan University of Technology-Materials Science Edition* **2005,** *20* (3), 31-34.

26. Holmberg, J. P. Hydrolytic Synthesis and Physicochemical Properties of TiO_2 Nanoparticles: Fundamentals and Applications. Department of Materials and Environmental Chemistry, Stockholm University, Sweden, 2012.
27. Moghaddam, H. M.; Nasirian, S., Ultrasonic wave effects on the diameter of TiO_2 nanoparticles. *South African Journal of Science* **2011,** *107* (3-4), 01-05.
28. Noguchi, S., Predictive factors for response to docetaxel in human breast cancers. *Cancer science* **2006,** *97* (9), 813-820.
29. Mathew, A.E.; Mejillano, M. R.; Nath, J. P.; Himes, R. H.; Stella, V. J. *J. Med. Chem.* 1992, *35*, 145-151.
30. Crown, J.; O'Leary, M.; Ooi, W.-S., Docetaxel and PCT in the treatment of breast cancer: a review of clinical experience. *The oncologist* **2004,** *9* (Supplement 2), 24-32.
31. Stoller, M.; Miranda, L.; Chianese, A., Optimal feed location in a spinning disc reactor for the production of TiO_2 nanoparticles. *Chemical Engineering Transactions* **2009,** *17*, 993-998.
32. Vijayalakshmi, R.; Rajendran, V., Synthesis and characterization of nano-TiO_2 via different methods. *Arch App Sci Res* **2012,** *4* (2), 1183-1190.
33. Holmberg, J. P. Hydrolytic Synthesis and Physicochemical Properties of TiO_2 Nanoparticles: Fundamentals and Applications. Department of Materials and Environmental Chemistry, Stockholm University, Sweden, 2012.
34. Filipe, V.; Hawe, A.; Jiskoot, W., Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharmaceutical research* **2010,** *27* (5), 796-810.
35. Erdem, B.; Hunsicker, R. A.; Simmons, G. W.; Sudol, E. D.; Dimonie, V. L.; El-Aasser, M. S., XPS and FTIR surface characterization of TiO_2 particles used in polymer encapsulation. *Langmuir* **2001,** *17* (9), 2664-2669.
36. Instruments, M., Zeta potential: An introduction in 30 minutes. *Zetasizer Nano Serles Technical Note. MRK654-01* **2011**.
37. Cheow, W. S.; Hadinoto, K., Factors affecting drug encapsulation and stability of lipid–polymer hybrid nanoparticles. *Colloids and Surfaces B: Biointerfaces* **2011,** *85* (2), 214-220.
38. Vinogradov, S. V.; Bronich, T. K.; Kabanov, A. V., Nanosized cationic hydrogels for drug delivery: preparation, properties and interactions with cells. *Advanced drug delivery reviews* **2002,** *54* (1), 135-147

39. Levin, S., High Performance Liquid Chromatography (HPLC) in the Pharmaceutical Analysis. *Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing* **2010**.
40. Zubay, G., *Biochemistry*. 3rd ed.; Wm. C. Brown: Dubuque, Iowa, 1993.
41. Long, T. C.; Saleh, N.; Tilton, R. D.; Lowry, G. V.; Veronesi, B., Titanium Dioxide (P25) produces Reactive Oxygen Species in Immortalized Brain Microglia (BV2): Implications for Nanoparticle Neurotoxicity. Environmental Science and Technology 2006, 40, (14), 4346-4352.
42. Vileno, B.; Lekka, M.; Sienkiewicz, A.; Jeney, S.; Stoessel, G.; Lekki, J.; Forró, L.; Stachura, Z., Stiffness Alterations of Single Cells Induced by UV in the Presence of NanoTiO_2. Environmental Science and Technology 2007, 41, (14), 5149-5153.
43. Longley, D.; Johnston, P., Molecular mechanisms of drug resistance. *The Journal of pathology* **2005,** *205* (2), 275-292.
44. Cho, K.; Wang, X.; Nie, S.; Shin, D. M., Therapeutic nanoparticles for drug delivery in cancer. *Clinical Cancer Research* **2008,** *14* (5), 1310-1316.
45. Lu, K.-H.; Lue, K.-H.; Chou, M.-C.; Chung, J.-G., Paclitaxel induces apoptosis via caspase-3 activation in human osteogenic sarcoma cells (U-2 OS). *Journal of orthopaedic research* **2005,** *23* (5), 988-994.
46. Pozarowski, P.; Darzynkiewicz, Z., Analysis of cell cycle by flow cytometry. In *Checkpoint Controls and Cancer*, Springer: 2004; pp 301-311.